SYSTEM FOR REAL TIME DETECTION OF NUCLEIC ACID AMPLIFICATION PRODUCTS

The invention relates generally to the field of nucleic acid amplification, and more particularly to a system for measuring in real time polynucleotide products from nucleic acid amplification processes, such as polymerase chain reaction (PCR).

Background

Nucleic acid sequence analysis is becoming increasingly important in many research, medical, and industrial fields, e.g. Caskey, Science 236: 1223-1228 (1987); Landegren et al, Science, 242: 229-237 (1988); and Arnheim et al, Ann. Rev. Biochem., 61: 131-156 (1992). The development of several nucleic acid amplification schemes has played a critical role in this trend, e.g. polymerase chain reaction (PCR), Innis et al, editors, PCR Protocols (Academic Press, New York, 1990); McPherson et al, editors, PCR: A Practical Approach (IRL Press, Oxford, 1991); ligation-based amplification techniques, Barany, PCR Methods and Applications 1: 5-16 (1991); and the like.

PCR in particular has become a research tool of major importance with applications in cloning, analysis of genetic expression, DNA sequencing, genetic mapping, drug discovery, and the like, e.g. Arnheim et al (cited above); Gilliland et al, Proc. Natl. Acad. Sci., 87: 2725-2729 (1990); Bevan et al, PCR Methods and Applications, 1: 222-228 (1992); Green et al, PCR Methods and Applications, 1: 77-90 (1991); Blackwell et al, Science, 250: 1104-1110 (1990).

A wide variety of instrumentation has been developed for carrying out nucleic acid amplifications, particularly PCR, e.g. Johnson et al, U.S. patent 5,038,852 (computer-controlled thermal cycler); Wittwer et al, Nucleic Acids Research, 17: 4353-4357 (1989)(capillary tube PCR); Hallsby, U.S. patent 5,187,084 (air-based temperature control); Garner et al, Biotechniques, 14: 112-115 (1993)(high-throughput PCR in 864-well plates); Wilding et al, International application No.

PCT/US93/04039 (PCR in micro-machined structures); Schnipelsky et al, European patent application No. 90301061.9 (publ. No. 0381501 A2)(disposable, single use PCR device), and the like. Important design goals fundamental to PCR instrument development have included fine temperature control, minimization of sample-to-

5   sample variability in multi-sample thermal cycling, automation of pre- and post-PCR processing steps, high speed cycling, minimization of sample volumes, real time measurement of amplification products, minimization of cross-contamination, or sample carryover, and the like. In particular, the design of instruments that permit PCR to be carried out in closed reaction chambers and monitored in real time is highly

10   desirable. Closed reaction chambers are desirable for preventing cross-contamination, e.g. Higuchi et al, Biotechnology, 10: 413-417 (1992) and 11: 1026-1030 (1993); and Holland et al, Proc. Natl. Acad. Sci., 88: 7276-7280 (1991). Clearly, the successful realization of such a design goal would be especially desirable in the analysis of diagnostic samples, where a high frequency of false positives and false negatives would

15   severely reduce the value of the PCR-based procedure. Real time monitoring of a PCR permits far more accurate quantitation of starting target DNA concentrations in multiple-target amplifications, as the relative values of close concentrations can be resolved by taking into account the history of the relative concentration values during the PCR. Real time monitoring also permits the efficiency of the PCR to be evaluated,

20   which can indicate whether PCR inhibitors are present in a sample.

Holland et al (cited above) and others have proposed fluorescence-based approaches to provide real time measurements of amplification products during a PCR. Such approaches have either employed intercalating dyes (such as ethidium bromide) to indicate the amount of double stranded DNA present, or they have employed probes

25   containing fluorescer-quencher pairs (the so-called "Tac-Man" approach) that are cleaved during amplification to release a fluorescent product whose concentration is proportional to the amount of double stranded DNA present.

Unfortunately, successful implementation of these approaches has been impeded because the required fluorescent measurements must be made against a very

30   high fluorescent background. Thus, even minor sources of instrumental noise, such as the formation of condensation in the chamber during heating and cooling cycles, formation of bubbles in an optical path, particles or debris in solution, differences in sample volumes--and hence, differences in signal emission and absorbence, and the like, have hampered the reliable measurement of the fluorescent signals.

35   In view of the above, it would be advantageous if an apparatus were available which permitted stable and reliable real time measurement of fluorescent indicators of amplification products resulting from any of the available nucleic acid amplification schemes.

Summary of the Invention

The invention relates to a system for carrying out real time fluorescence-based measurements of nucleic acid amplification products. In a preferred embodiment of the invention, an excitation beam is focused into a reaction mixture containing (i) a first fluorescent indicator capable of generating a first fluorescent signal whose intensity is proportional to the amount of an amplification product in the volume of the reaction mixture illuminated by the excitation beam and (ii) a second fluorescent indicator homogeneously distributed throughout the reaction mixture and capable of generating a second fluorescent signal proportional to the volume of reaction mixture illuminated by the excitation beam. It is understood that the proportionality of the fluorescent intensities is for a constant set of parameters such as temperature, pH, salt concentration, and the like, that independently influence the fluorescent emissions of organic dyes.

Preferably, the excitation beam is focused into the reaction mixture by a lens through a portion of a wall of a closed reaction chamber containing the reaction mixture. In further preference, the same lens collects the first and second fluorescent signals generated by the first and second fluorescent indicators, respectively, in response to the excitation beam; thus, variability in the collected signal due to misalignment of excitation and collection optics is avoided. In this embodiment, whenever the lens directs the excitation beam through a portion of a wall of the closed reaction chamber which is not in contact with the reaction mixture, that portion of the wall is heated so that condensation from the reaction mixture does not form in the optical pathway of the fluorescent signals being collected by the lens, thereby removing another source of variability in the collected signal.

In the most preferred embodiment, the reaction chamber is a tube with a closed end, referred to herein as the bottom of the tube, and an open end, referred to herein as the top of the tube, which can be closed with a cap such that a leak-proof seal is formed. In other words, once a reaction mixture is placed in the tube and the cap is attached, a closed reaction chamber is formed. In this most preferred embodiment, (1) the reaction mixture fills a portion of the tube, generally at the bottom of the tube, such that a void is left between the cap of the tube and a top surface of the reaction mixture, (2) the walls of the tube are frosted; that is, they are made of a material that transmits and scatters light, and (3) the lens without contacting the cap focuses the excitation beam through the cap into the reaction mixture through its top surface and collects the resulting fluorescence generated by the first and second fluorescent indicators. As mentioned above, the portion of the tube through which the excitation beam passes--the cap in this embodiment--is heated to prevent the formation of

condensation which would introduce an added source of variability in the measurement of the collected fluorescent signals. Potential variability that could arise from sequential analysis of the first and second fluorescent signals is eliminated by simultaneously analyzing the signals by spectrally separating the signal light onto an

5   array of photo detectors, e.g. by diffracting the signal onto a charged-coupled device (CCD) array.

As discussed more fully below, an excitation beam generated by a single light source, e.g. a laser, is conveniently distributed to a plurality of closed reaction chambers by fiber optics. Likewise, the same fiber optics can collect the fluorescent

10   signals from the plurality of reaction chambers for analysis by a single detection and analysis system.

Preferably, the system is employed with the PCR amplification of nucleic acids.

The system of the invention permits accurate real time monitoring of nucleic amplification reactions by providing apparatus and fluorescent reagents for generating

15   a stable fluorescent signal proportional to the amount of amplification product and independent of variations in the volume of reaction mixture. The availability of data showing the progress of amplification reactions leads to more accurate estimates of relative starting concentrations of target nucleic acids, to rapid assessment of the efficiency of the amplification reactions, and opens the possibility of reduced reagent

20   usage and feedback reaction control.

Brief Description of the Figures

Figure 1 diagrammatically illustrates a preferred embodiment of the sample

25   interface components of the system of the invention.

Figure 2 diagrammatically illustrates a preferred embodiment for simultaneously monitoring a plurality of amplification reactions by sequentially interrogating reactions via a fiber optic multiplexer.

Figure 3 shows spectrally separated fluorescent intensity data for a

30   tetramethylrhodamine fluorescent indicator, a fluorescein fluorescent indicator, and instrument background registered by a CCD array of the preferred embodiment described below.

Figure 4 shows the time dependence of fluorescent signals from a fluorescein dye proportional to the amplification product (first fluorescent indicator) and a

35   tetramethylrhodamine dye employed as a second fluorescent indicator during a typical PCR.

Figure 5 shows the cycle dependence of the ratio of the intensities of the fluorescein and tetramethylrhodamine dyes from the same PCR whose time dependent data is shown in Figure 3.

Figure 6 shows data relating the amount of amplification product to cycle
5   number in separate PCRs having different starting concentrations of the same target nucleic acid.

Definitions

As used herein, the term "stable" in reference to a fluorescent signal means that
10   the root means square (RMS) deviation in the signal due to noise is less than or equal to two percent of the average signal magnitude. More preferably, stable means that the RMS deviation in the signal due to noise is less than or equal to one percent of the average signal magnitude.

15   Detailed Description of the Invention

The invention is a fluorescence-based system for monitoring in real time the progress of a nucleic acid amplification reaction. The type of amplification scheme used with the system is not critical, but generally the system requires either the use of a nucleic acid polymerase with exonuclease activity or a population of double stranded
20   DNA which increases during the course of the reaction being monitored. Exemplary amplification schemes that may be employed with the system of the invention include PCR, ligase-based amplification schemes, such as ligase chain reaction (LCR), Q-beta replicase-based amplification schemes, strand displacement amplification (SDA) schemes, such as described by Walker et al, Nucleic Acids Research, 20: 1691-1696
25   (1992), and the like. A comprehensive description of nucleic acid amplification schemes is provided by Keller and Manak, DNA Probes, Second Edition (Stockton Press, New York, 1993). Fundamental to the system is the measurement of ratios of fluorescent intensities of a first fluorescent indicator and an internal standard, referred to herein as a second fluorescent indicator. The first and second fluorescent indicators
30   must be spectrally resolvable. That is, their respective emission spectra must be sufficiently non-overlapping so that separate emission peaks are observed in the combined spectrum. Clearly, the system may be generalized to include a plurality of first fluorescent indicators, e.g. to monitor the simultaneous amplification of several target nucleic acids in a single reaction, so that a plurality of fluorescent intensity ratios
35   are monitored. Several spectrally resolvable dyes suitable for use in such embodiments are disclosed in Fung et al, U.S. patent 4,855,225; Menchen et al, U.S. patent 5,188,934; Bergot et al, International Application PCT/US90/05565; and like references.

The system includes a sample interface--that is, optical components operationally associated with a closed reaction chamber--which comprises a lens for focusing an excitation beam into the reaction mixture and for collecting the resulting fluorescence and a fiber optic for transmitting both the excitation beam from a light

5   source to the lens and the fluorescent signals from the lens to a detection and analysis means. Preferably, the reaction mixture is contained in a closed reaction chamber to prevent cross-sample contamination, or so-called "carryover." The lens therefore focuses the excitation beam and collects fluorescence through a portion of a wall of the closed reaction chamber. As mentioned above, the preferred reaction chamber is a

10   tube, e.g. having the geometry and volume of a conventional Eppendorf tube. The tube is closed after the reaction mixture is added by attaching a cap to the open end of the tube. In a preferred embodiment of the sample interface for PCR, the lens directs the excitation beam and collects fluorescence through the cap of the tube, as illustrated in Figure 1. In the illustrated configuration, a first end fiber optic 2 is held by ferrule

15   4, housing 6, and plate 10 in a co-axial orientation with lens 8. A second end of fiber optic 2 (not shown) is operationally associated with a light source and detection and analysis means, discussed more fully below. The distance between the end face of fiber optic 2 and lens 8 is determined by several factors, including the numerical aperture of the fiber optic, the geometry of tube 18, the focal length of lens 8, the

20   diameter of lens 8, and the like. Guidance for selecting values for such variables in any particular embodiment is readily found in standard texts on optical design, e.g. Optics Guide 5 (Melles Griot, Irvine, CA, 1990), or like reference. In the illustrated embodiment, lens 8 has a diameter of 8 mm and is composed of material BK7, available from Edmund Scientific (Barrington, NJ). Fiber optic 2 has a numerical

25   aperture of .2. Preferably, the design permits maximal transmission of excitation beam 28 to reaction mixture 22. For example, lens 8, numerical aperture of fiber optic 2, and the distance between the end of fiber optic 2 and lens 8 are selected so that the diameter of lens 8 equals or exceeds the diameter of excitation beam 28 where beam 28 impinges on the lens (as illustrated in Figure 1). Excitation beam 28 is focused

30   through cap 16, void 24, and top surface 26 of reaction mixture 22 to a region approximately 1-3 times the diameter of the fiber optic just below, e.g. 1-3 mm, surface 26. This degree of focusing is not a critical feature of the embodiment; it is a consequence of adapting the sample interface to the geometry and dimensions of a sample holder of a commercially available thermal cycler. In other embodiments, the

35   geometry and dimension may permit a sharper focus into the reaction mixture.

The lens of the invention may have a variety of shapes depending on particular embodiments. For example, the lens may be a sphere, truncated sphere, cylinder, truncated cylinder, oblate spheroid, or truncated oblate spheroid, or the like, and may

be composed of any suitably transparent refractive material, such as disclosed by Hlousek, U.S. patent 5,037,199; Hoppe et al, U.S. patent 4,747,87; Moring et al, U.S. patent 5,239,360; Hirschfield, U.S. patent 4,577,109; or like references.

5　　　Fluorescent light generated by excitation beam 28 is collected by lens 8 along approximately the same optical pathway as that defined by excitation beam 28 and focused onto the end of fiber optic 2 for transmission to optical separation and analysis components of the system.

In further preference, the sample interface also includes means for heating the portion of the wall of the reaction chamber used for optical transmission in order to

10　　reduce variability due to scatter and/or absorption of the excitation beam and signal from condensation of reaction mixture components. In the embodiment of Figure 1, the portion of the reaction chamber (tube 18) wall used for optical transmission is cap 16. Accordingly, heating element 12 and heat-conductive platen 14 are employed to heat cap 16. Preferably, heating element 12 comprises resistance heating elements and

15　　temperature sensors that permit programmed controlled of the temperature of cap 16. Cap 16 is maintained at a temperature above the condensation points of the components of the reaction mixture. Generally, cap 16 may be maintained at a temperature in the range of 94-110°C. Preferably, cap 16 is maintained at a temperature in the range of about 102°C to about 105°C since the principal solvent in

20　　the reaction mixture is usually water. More preferably, cap 16 is maintained at 103°C. Preferably, in embodiments employing thermal cycling, the cap-heating components described above are thermally isolated from heating-conducting component 20 employed to cyclically control the temperature of reaction mixture 22.

As mentioned above, walls of tube 18 are preferably frosted so that any

25　　spurious reflections from the walls of heat-conducting component 20 are diffused or scattered to reduce the contributions such reflections may make to the collected signal. Walls of ordinarily translucent or transparent tubes are conveniently frosted by etching or roughening.

Selection of appropriate materials for the components described above is well

30　　within the skill of an ordinary mechanical engineer. Exemplary criterion for material selection include (i) degree of thermal expansion, especially for amplification schemes employing thermal cycling, and its affect on the alignment of the optical components, (ii) optical transmission properties in the excitation wavelengths and fluorophore emission wavelengths employed, (iii) chemical inertness of the reaction chamber

35　　relative to components of the reaction mixture, (iv) degree to which critical reaction components, e.g. polymerases, target nucleic acids, would tend to adsorb onto chamber walls, (v) minimization of fluorescent materials in the optical pathway, and

the like.   Typically, tubes containing amplification reaction mixtures are made of
polypropylene or like materials.

    The sample interface shown in Figure 1 may be employed individually or it may
be employed as one of a plurality of identical interfaces in a single instrument, as

5   shown diagrammatically in Figure 2. In the illustrated embodiment, individual sample
interfaces 31, arrayed in holder 30 (which may, for example, be a heating block
associated with thermal cycler 32, such as described in Mossa et al, European patent
application No. 91311090.4, publ. No. 0488769 A2) are connected by fiber optics 34
to fiber optic multiplexer 36, which selectively permits transmission between individual

10   fiber optics and port 35, e.g under user control via a programmed microprocessor. In
a preferred configuration, excitation beam 41, generated by light source 52 and
controller 54, passes through beam splitter 40 and is focused onto port 35 by lens 38,
where it is sequentially directed by fiber optic multiplexer 36 to each of a
predetermined set, or subset, of fiber optics 34. Conversely, a fluorescent signal

15   generated in a reaction chambers is collected by lens 8 and focused onto a fiber optic
which, in turn, transmits the signal to a detection and analysis means, possibly via a
fiber optic multiplexer. Returning to Figure 2, a fluorescent signal collected by a
sample interface is directed to fiber optic multiplexer 36 where it emerges through port
35 and is collected and collimated by lens 38. Lens 38 directs the fluorescent signal to

20   beam splitter 40 which, in turn, selectively directs the signal through cut-off filter 42,
which prevents light from the excitation beam from reaching the signal detection
components. Beam splitter 40 may be a conventional dichroic mirror, a fully reflective
mirror with an aperture to pass the excitation beam (e.g. as disclosed in U.S. patent
4,577,109), or like component. After passing through cut-off filter 42, the fluorescent

25   signal is directed by lens 44 to a spectral analyzer which spectrally separates the
fluorescent signal and measures the intensities of a plurality of the spectral components
of the signal. Typically, a spectral analyzer comprises means for separating the
fluorescent signal into its spectral components, such as a prism, diffraction grating, or
the like, and an array of photo-detectors, such as a diode array, a charge-coupled

30   device (CCD) system, an array of bandpass filters and photomultiplier tubes, or the
like. In the preferred embodiment of Figure 2, the spectral analyzer comprises
diffraction grating 46 (e.g., model CP-140, Jobin-Yvon, NJ) and CCD array 48 (e.g.,
model S2135 Princeton Instruments, NJ), which is linked to CCD controller 50.

    An exemplary CCD array suitable for analyzing fluorescent signal from

35   fluorescein and tetramethylrhodamine is partitioned into 21 collection bins which span
the 500 nm to 650 nm region of the spectrum. Each bin collects light over a 8.5 nm
window. Clearly, many alternative configurations may also be employed. An

exemplary application of a CCD array for spectral analysis is described in Karger et al,
Nucleic Acids Research, 19: 4955-4962 (1991).

5   Analyzing the fluorescent signal based on data collected by a spectral analyzer
is desirable since components of the signal due to one or more first fluorescent
indicators and a second fluorescent indicator (from which intensity ratios are
calculated) can be analyzed simultaneously and without the introduction of
wavelength-specific system variability that might arise, e.g. by misalignment, in a
system based on multiple beam splitters, filters, and photomultiplier tubes. Also, a
spectral analyzer permits the use of "virtual filters" or the programmed manipulation of
10   data generated from the array of photo-detectors, wherein a plurality of discrete
wavelength ranges are sampled--in analogy with physical bandpass filters--under
programmable control via an associated microprocessor. This capability permits a high
degree of flexibility in the selection of dyes as first and second fluorescent indicators.

           Generally, the detection and analysis means may be any detection apparatus to
15   provides a readout that reflect the ratio of intensities of the signals generated by the
first and second fluorescent indicators. Such apparatus is well know in the art, as
exemplified by U.S. patents 4,577,109 and 4,786,886 and references such as The
Photonics Design & Applications Handbook, 39th Edition (Laurin Publishing Co.,
Pittsfield, MA, 1993).

20   Preferably, the system of the invention is employed to monitor PCRs, although
it may also be employed with a variety of other amplification schemes, such as LCR.
Descriptions of and guidance for conducting PCRs is provided in an extensive
literature on the subject, e.g. including Innis et al (cited above) and McPherson et al
(cited above). Briefly, in a PCR two oligonucleotides are used as primers for a series
25   of synthetic reactions that are catalyzed by a DNA polymerase. These
oligonucleotides typically have different sequences and are complementary to
sequences that (i) lie on opposite strands of the template, or target, DNA and (ii) flank
the segment of DNA that is to be amplified. The target DNA is first denatured by
heating in the presence of a large molar excess of each of the two oligonucleotides and
30   the four deoxynucleoside triphosphates (dNTPs). The reaction mixture is then cooled
to a temperature that allows the oligonucleotide primers to anneal to their target
sequences, after which the annealed primers are extended with DNA polymerase. The
cycle of denaturation, annealing, and extension is then repeated many times, typically
25-35 times. Because the products of one round of amplification serve as target
35   nucleic acids for the next, each successive cycle essentially doubles the amount of
target DNA, or amplification product.

           As mentioned above an important aspect of the invention is the fluorescent
dyes used as the first and second fluorescent indicators. By examining the ratio of the

fluorescent intensities of the indicators, the effects of most sources of systematic variability, which would be apparent in the intensities alone, are eliminated. Generally, in accordance with the invention, the first fluorescent indicator may be a complex-forming dye or a dye covalently attached to an oligonucleotide probe which is

5   degraded during polymerization steps to generate a signal. This later embodiment relates to the so-called "Tacman" approach, described by Holland et al, Proc. Natl. Acad. Sci., 88: 7276-7280 (1991). As used herein, the term "complex-forming" in reference to a dye means that a dye is capable of forming a stable non-covalent complex with either double stranded or triple stranded nucleic acid structures, usually

10   DNA, and that the dye's fluorescent characteristics are substantially different in the complexed state as compared to a non-complexed, i.e. usually free-solution, state. Preferably, the quantum efficiency of fluorescence of an complex-forming dye is enhanced in the complexed state as compared to the free-solution state, thereby resulting in enhanced fluorescent upon complex formation. Exemplary complex-

15   forming dyes include ethidium bromide, propidium iodide, thiazole orange, acridine orange, daunomycin, mepacrine, 4',6'-diaminidino-2-phenylindole (DAPI), oxazole orange, bisbenzimidaxole dyes, such as Hoechst 33258 and Hoechst 33342, and heterodimers of various intercalating dyes, such as ethidium, acridine, thiazolium, and oxazolium dyes (known by their acronyms POPRO, BOPRO, YOPRO, and TOPRO),

20   and like dyes, which are described in the following references: Haugland, pgs. 221-229 in Handbook of Fluorescent Probes and Research Chemicals, 5th Edition (Molecular Probes, Inc., Eugene, 1992); Glazer et al, Proc. Natl. Acad. Sci., 87: 3851-3855 (1990); Srinivasan et al, Applied and Theoretical Electrophoresis, 3: 235-239 (1993); Kapuscinski et al, Anal. Biochem., 83: 252-257 (1977); Hill, Anal. Biochem.,

25   70: 635-638 (1976); Setaro et al, Anal. Biochem., 71: 313-317 (1976); and Latt et al, J. Histochem. Cytochem., 24:24-33 (1976); and Rye et al, Nucleic Acids Research, 20: 2803-2812 (1992). Preferably, when complex-forming dyes are employed as first fluorescent indicators, such dyes are selected from the group consisting of thiazole orange, ethidium bromide, and TOPRO.

30   Dyes employed as second fluorescent indicators include fluorescent dyes whose fluorescent characteristics are substantially unaffected by the presence or association with nucleic acids, particularly double stranded DNA. Such dyes may include virtually any fluorescent dye fulfilling this criterion which is also spectrally resolvable from whatever first fluorescent indicators that are employed. Preferred second fluorescent

35   indicators include rhodamine dyes and fluorescein dyes. More preferably, the second fluorescent indicator is tetramethylrhodamine or 2',4',5',7',-tetrachloro-4,7-dichlorofluorescein, the latter being disclosed by Menchen et al, U.S. patent 5,188,934.

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(54) Title: SYSTEM FOR REAL TIME DETECTION OF NUCLEIC ACID AMPLIFICATION PRODUCTS

(57) Abstract

A system is provided for carrying out real time fluorescence-based measurements of nucleic acid amplification products. In a preferred embodiment of the invention, an excitation beam is focused into a reaction mixture through a surface, the reaction mixture containing: (i) a first fluorescent indicator capable of generating a first fluorescent signal whose intensity is proportional to the amount of an amplification product in the volume of the reaction mixture illuminated by the excitation beam and (ii) a second fluorescent indicator homogeneously distributed throughout the reaction mixture capable of generating a second fluorescent signal proportional to the volume of reaction mixture illuminated by the excitation beam. Preferably, the excitation beam is focused into the reaction mixture by a lens through a portion of a wall of a closed reaction chamber containing the reaction mixture. The same lens is used to collect the first and second fluorescent signals generated by the first and second fluorescent indicators, respectively, in response to the excitation beam. The ratio of the fluorescent intensities of the first and second fluorescent signals provides a stable quantitative indicator of the amount of amplification product synthesized in the course of the amplification reaction.

In a preferred embodiment, a first fluorescent indicator and a second fluorescent indicator are both covalently attached to an oligonucleotide probe as described by Lee et al, Nucleic Acid Research, 21: 3761-3766 (1993). More specifically, fluorescein is used as the first fluorescent indicator and

5   tetramethylrhodamine is used as the second fluorescent indicator such that the tetramethylrhodamine moiety substantially quenches any fluorescent emissions by the fluorescein moiety. Thus, when both dyes are attached to the same oligonucleotide, only the tetramethylrhodamine is capable of generating a fluorescent signal. When the oligonucleotide is cleaved, e.g. via the 5'->3' exonuclease activity of a DNA

10   polymerase, separating the two dyes, the fluorescein become capable of generating a fluorescent signal. Preferably, in this embodiment, the excitation beam is generated from the 488 nm emission line of an argon ion laser. In accordance with the invention, in a PCR the production of "free" fluorescein in this embodiment is proportional to the amount of DNA synthesis catalyzed by the DNA polymerase employed, and hence, the

15   amount of amplification product. In this embodiment, preferably the first fluorescent indicator is fluorescein, e.g. 6-FAM (available from Applied Biosystems, Foster City), and the second fluorescent indicator is either tetramethylrhodamine or 2',4',5',7',-tetrachloro-4,7-dichlorofluorescein.

Such oligonucleotide probes of the invention can be synthesized by a number of

20   approaches, e.g. Ozaki et al, Nucleic Acids Research, 20: 5205-5214 (1992); Agrawal et al, Nucleic Acids Research, 18: 5419-5423 (1990); or the like. Preferably, the oligonucleotide probes are synthesized on an automated solid phase DNA synthesizer using phosphoramidite chemistry, e.g. Applied Biosystems, Inc. model 392 or 394 DNA synthesizer (Foster City, CA). The first and second fluorescent indicators can be

25   covalently attached to predetermined nucleotide of an oligonucleotide by using nucleoside phosphoramidite monomers containing reactive groups. For example, such reactive groups can be on a phosphate, or phosphate analog, e.g. Agrawal et al (cited above), on the 5' hydroxyl when attachment is to the 5' terminal nucleotide, e.g. Fung et al, U.S. patent 4,757,141 or Hobbs Jr., U.S. patent 4,997,928, and on base moieties, e.g. as

30   disclosed by Ruth, U.S. patent 4,948,882; Haralambidis et al, Nucleic Acids Research, 15: 4857-4876 (1987); Urdea et al, U.S. patent 5,093,232; Cruickshank U.S. patent 5,091,519; Hobbs Jr. et al, U.S. patent 5,151,507; or the like. Most preferably, nucleotides having pyrimidine moieties are derivatized. In further preference, the 3' terminal nucleotide of the oligonucleotide probe is blocked or rendered incapable of

35   extension by a nucleic acid polymerase. Such blocking is conveniently carried out by the attachment of a phosphate group, e.g. via reagents described by Horn and Urdea, Tetrahedron Lett., 27: 4705 (1986), and commercially available as 5' Phosphate-ON™ from Clontech Laboratories (Palo Alto, California). Preferably, the oligonucleotide probe

is in the range of 15-60 nucleotides in length. More preferably, the oligonucleotide probe
is in the range of 18-30 nucleotides in length.

The separation of the first and second fluorescent indicators within the
oligonucleotide probe can vary depending on the nature of the first fluorescent indicator

5   and second fluorescent indicator, the manner in which they are attached, the illumination
source, and the like. Guidance concerning the selection of an appropriate distance for a
given embodiment is found in numerous references on resonant energy transfer between
fluorescent molecules and quenching molecules (also sometimes referred to as "donor"
molecules and "acceptor" molecules, respectively), e.g. Stryer and Haugland, Proc. Natl.

10   Acad. Sci., 58: 719-726 (1967); Clegg, Meth. Enzymol., 211: 353-388 (1992); Cardullo et
al, Proc. Natl. Acad. Sci., 85: 8790-8794 (1988); Ozaki et al (cited above); Haugland
(cited above); Heller et al, Fed. Proc., 46: 1968 (1987); and the like. The first and second
fluorescent indicators must be close enough so that substantially all, e.g. 90%, of the
fluorescence from the first fluorescent indicator is quenched. Typically, for energy

15   transfer-based quenching, the distance between the first and second fluorescent indicators
should be within the range of 10-100 angstroms. In one embodiment, the first and second
fluorescent indicators are separated by between about 4 to 10 nucleotides, and more
preferably, they are separated by between 4 and 6 nucleotides, with the proviso that there
are no intervening secondary structures, such as hairpins, or the like. Preferably, either the

20   first or second fluorescent indicator is attached to the 5' terminal nucleotide of the
oligonucleotide probe.

In another embodiment, an oligonucleotide probe is provided with first and
second fluorescent indicators attached at opposite ends. This configuration
permits a more conveniently synthesized probe that in free solution forms a random

25   coil bringing the first and second fluorescent indicators within energy transfer
range. Thus, in the non-hybridized state, the excited fluorescent moiety is
quenched. In the hybridized state the fluorescent moiety and quencher are drawn
apart so that energy transfer become negligible permitting the excited fluorescent
moiety to emit fluorescence.

30   There are many linking moieties and methodologies for attaching indicator
molecules to the 5' or 3' termini of oligonucleotides, as exemplified by the
following references: Eckstein, editor, Oligonucleotides and Analogues: A
Practical Approach (IRL Press, Oxford, 1991); Zuckerman et al, Nucleic Acids
Research, 15: 5305-5321 (1987)(3' thiol group on oligonucleotide); Sharma et al,

35   Nucleic Acids Research, 19: 3019 (1991)(3' sulfhydryl); Giusti et al, PCR Methods
and Applications, 2: 223-227 (1993) and Fung et al, U.S. patent 4,757,141 (5'
phosphoamino group via Aminolink™ II available from Applied Biosystems,
Foster City, CA); Stabinsky, U.S. patent 4,739,044 (3' aminoalkylphosphoryl

group); Agrawal et al, Tetrahedron Letters, 31: 1543-1546 (1990)(attachment via
phosphoramidate linkages); Sproat et al, Nucleic Acids Research, 15: 4837
(1987)(5' mercapto group); Nelson et al, Nucleic Acids Research, 17: 7187-7194
(1989)(3' amino group); and the like.

5   Preferably, commercially available linking moieties are employed that can
be attached to an oligonucleotide during synthesis, e.g. available from Clontech
Laboratories (Palo Alto, CA).

         Rhodamine and fluorescein dyes are also conveniently attached to the 5'
hydroxyl of an oligonucleotide at the conclusion of solid phase synthesis by way of
10   dyes derivatized with a phosphoramidite moiety, e.g. Woo et al, U.S. patent
5,231,191; and Hobbs, Jr. U.S. patent 4,997,928.

         Clearly, related embodiments of the above may be employed wherein the first
fluorescent indicator is attached to an oligonucleotide probe with another non-
fluorescent quenching molecule, instead of a second fluorescent indicator. In such
15   embodiments, the second fluorescent indicator could be virtually any spectrally
resolvable fluorescent dye that did not interact with the amplification products.

                              EXPERIMENTAL

20

         Real time monitoring of PCR amplification of DNA encoding β-actin
                  from various starting concentrations of target DNA

         A 296 basepair segment of a target DNA encoding human β-actin was
amplified by PCR from various starting amounts in the range of 5×10^3 to 1×10^6 copies
25   of target DNA. The following primers and probe were employed:

                     5'-TCACCCACACTGTGCCCATCTACGA
                              (forward primer)

30   5'-CAGCGGAACCGCTCATTGCCAATGGT
                              (reverse primer)

              5'-A(FAM)TGCCCT(TMR)CCCCCATGCCATCCTGCGT
                                 (probe)

35

         wherein "FAM" indicates a fluorescein molecule coupled to the oligonucleotide by
reacting an NHS-ester group attached to the fluorescein's 6 carbon with a 5'-
aminophosphate attached to the 5'-terminal deoxyadenosine of the oligonucleotide in

                                    - 13 -

accordance with Fung et al, U.S. patent 5,212,304; and wherein "TMR" indicates a tetramethylrhodamine molecule coupled to the base moiety of the adjacent thymidine via the amino linking agent disclosed by Urdea et al, U.S. patent 5,093,232.

       PCRs were carried out in 0.2 mL MicroAmp tubes (Perkin-Elmer, Norwalk,
5   CT) with the following components: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 200 µM each of the nucleoside triphosphates (with dUTP substituted for dTTP in accordance with U.S. patent 5,035,996 to prevent carryover contamination), 300 nM each of forward and reverse primers, AmpliTaq (Perkin-Elmer, Norwalk, CT) at 0.05 U/µL. To this mixture was added 5 µL Raji DNA (Applied Biosystems, Foster City,
10   CA) at 10 ng/µL, 5 µL probe at 2 µM, and 1 µL uracil N-glycosylase at 1 unit/µL to bring the reaction volume to 51 µL. Heating and cooling cycles were carried out in a model 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT) fitted with a sample holder cover containing the sample interface components of the invention. The following temperature profile was employed: hold for 2 minutes at 50°C; hold for 10 minutes at
15   95°C; cycle through the following temperatures 40 times: 92°C for 15 seconds, 54°C for 15 seconds, 72°C for 1 minute; then hold at 72°C.

       Figure 3 illustrates data showing the emission spectra of the fluorescein and tetramethylrhodamine dyes employed as indicators above and fluorescence due to extraneous sources in the system.
20   Figure 4 illustrates data showing fluorescein fluorescent intensity and tetramethylrhodamine fluorescent intensity as a function of cycle number. The high frequency oscillations in intensity reflect the temperature dependence of the fluorescent emission of the two dyes. An increase in base line fluorescence for both dyes between cycles 10 and 28 is a system-based variation. In Figure 5, which illustrates the ratio of
25   fluorescein-to-tetramethylrhodamine fluorescent intensity from the same data, the system-based variation is eliminated and the RMS of fluctuations in the readout signal, that is, the ratio of fluorescent intensities, is less than 1% of the average magnitude of the measured ratio.

       Figure 6 illustrates data from PCR of the β-actin DNA starting from amounts
30   ranging from 5000 target molecules to 10^6 target molecules as indicated in the figure.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: System for real time detection
of nucleic acid amplification products

(iii) NUMBER OF SEQUENCES: 3

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(v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: 3.5 inch diskette
        (B) COMPUTER: IBM compatible
        (C) OPERATING SYSTEM: Windows 3.1/DOS 5.0
        (D) SOFTWARE: Microsoft Word for Windows, vers. 2.0

(vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/235,411
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 nucleotides
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCACCCACAC TGTGCCCATC TACGA   25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 26 nucleotides
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGCGGAACC GCTCATTGCC AATGGT   26

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 26 nucleotides
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGCCCTCCC CCATGCCATC CTGCGT   26

We claim:

1.   A system for monitoring in real time the progress of a nucleic acid
amplification reaction, the system comprising:

5   a reaction mixture for carrying out the nucleic acid amplification reaction so
that an amplification product is synthesized;

       a sample interface comprising a fiber optic co-axially disposed with a lens so
that an excitation beam transmitted by the fiber optic is focused into a volume of the
reaction mixture by the lens;

10   a first fluorescent indicator capable of generating a first fluorescent signal
whose intensity is proportional to the amount of an amplification product in the
volume of the reaction mixture illuminated by the excitation beam; and

       a second fluorescent indicator homogeneously distributed throughout the
reaction mixture and capable of generating a second fluorescent signal proportional

15 to the volume of reaction mixture illuminated by the excitation beam;

       wherein the lens of the sample interface collects a portion of the first
fluorescent signal and the second fluorescent signal and focuses said portion onto
the fiber optic, the fiber optic transmitting said portion to a detection and analysis
means.

20

2.   The system of claim 1 wherein said first fluorescent signal has an intensity
and said second fluorescent signal has an intensity, and wherein said detection and
analysis means provides a readout comprising a ratio of the intensities of said first
fluorescent signal and said second fluorescent signal.

25

.3.   The system of claim 2 wherein said first fluorescent indicator is a complex-
forming dye.

4.   The system of claim 3 wherein said system comprises a plurality of said
30 sample interfaces, each said fiber optic of said sample interface having a first end co-
axially disposed with said lens and a second end coupled to a fiber optic multiplexer.

5.   The system of claim 3 wherein said reaction mixture is contained in a closed
reaction vessel, said lens focusing said excitation beam through a portion of a wall of
35 the closed reaction vessel into said reaction mixture, and said portion of the wall
being heated so that condensation of components of said reaction mixture thereon is
prevented.

6.   The system of claim 5 wherein said closed reaction vessel is a frosted tube.

7.   The system of claim 3 wherein said system comprises a plurality of said first fluorescent indicators each corresponding to a different said amplification product.

5

8.   The system of claim 3 wherein said amplification reaction is a polymerase chain reaction.

9.   The system of claim 3 wherein said amplification reaction is a ligase chain
10   reaction

10.   The system of claim 2 wherein said amplification reaction is a polymerase chain reaction and wherein said first fluorescent indicator and said second fluorescent indicator are covalently attached to an oligonucleotide having a
15   nucleotide sequence complementary to a portion of a strand of said amplification product, said second fluorescent indicator quenching the fluorescence of said first fluorescent indicator.

11.   The system of claim 10 wherein said system comprises a plurality of said
20   sample interfaces, each said fiber optic of said sample interface having a first end co-axially disposed with said lens and a second end coupled to a fiber optic multiplexer.

12.   The system of claim 10 wherein said reaction mixture is contained in a closed reaction vessel, said lens focusing said excitation beam through a portion of a wall of
25   the closed reaction vessel into said reaction mixture, and said portion of the wall being heated so that condensation of components of said reaction mixture thereon is prevented.

13.   The system of claim 12 wherein said closed reaction vessel is a frosted tube.
30
14.   The system of claim 10 wherein said system comprises a plurality of said first fluorescent indicators each corresponding to a different said amplification product.
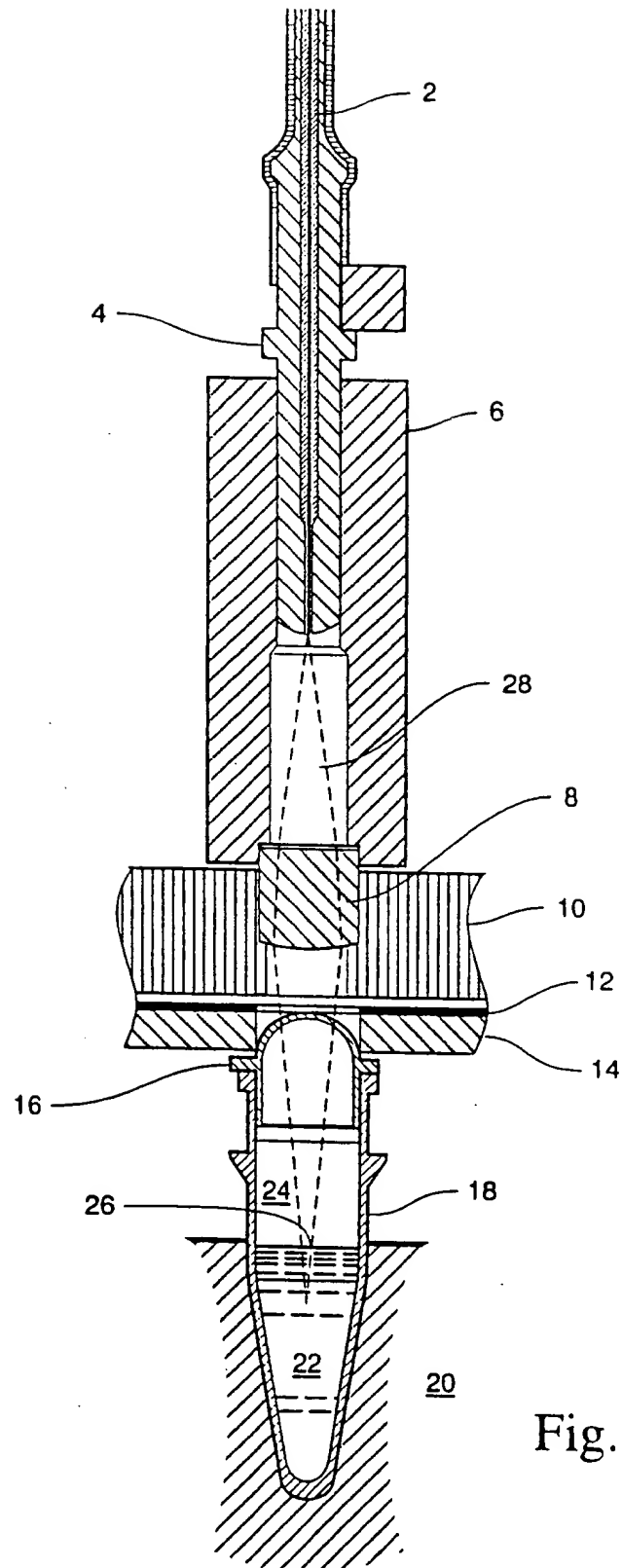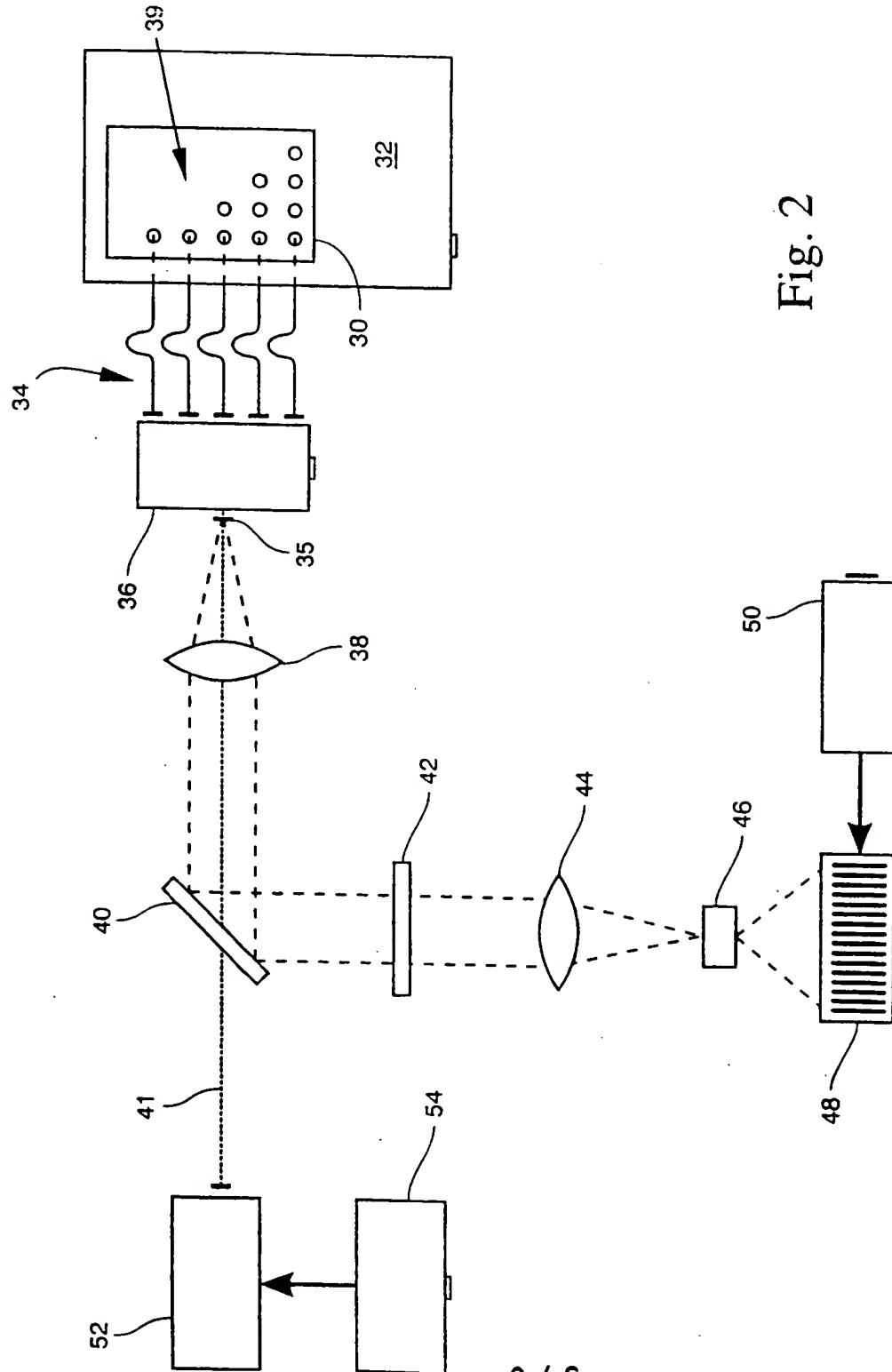
Fig. 1

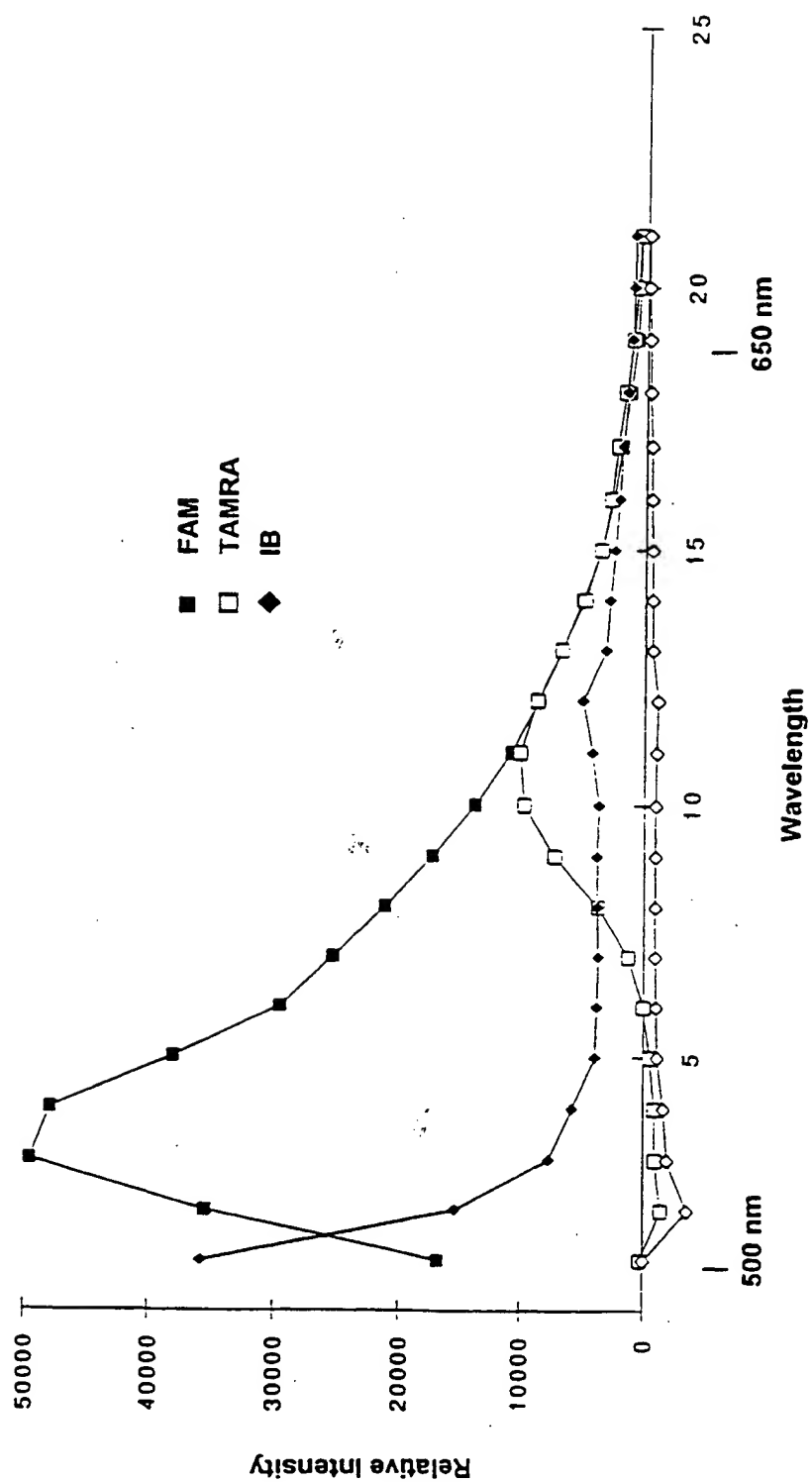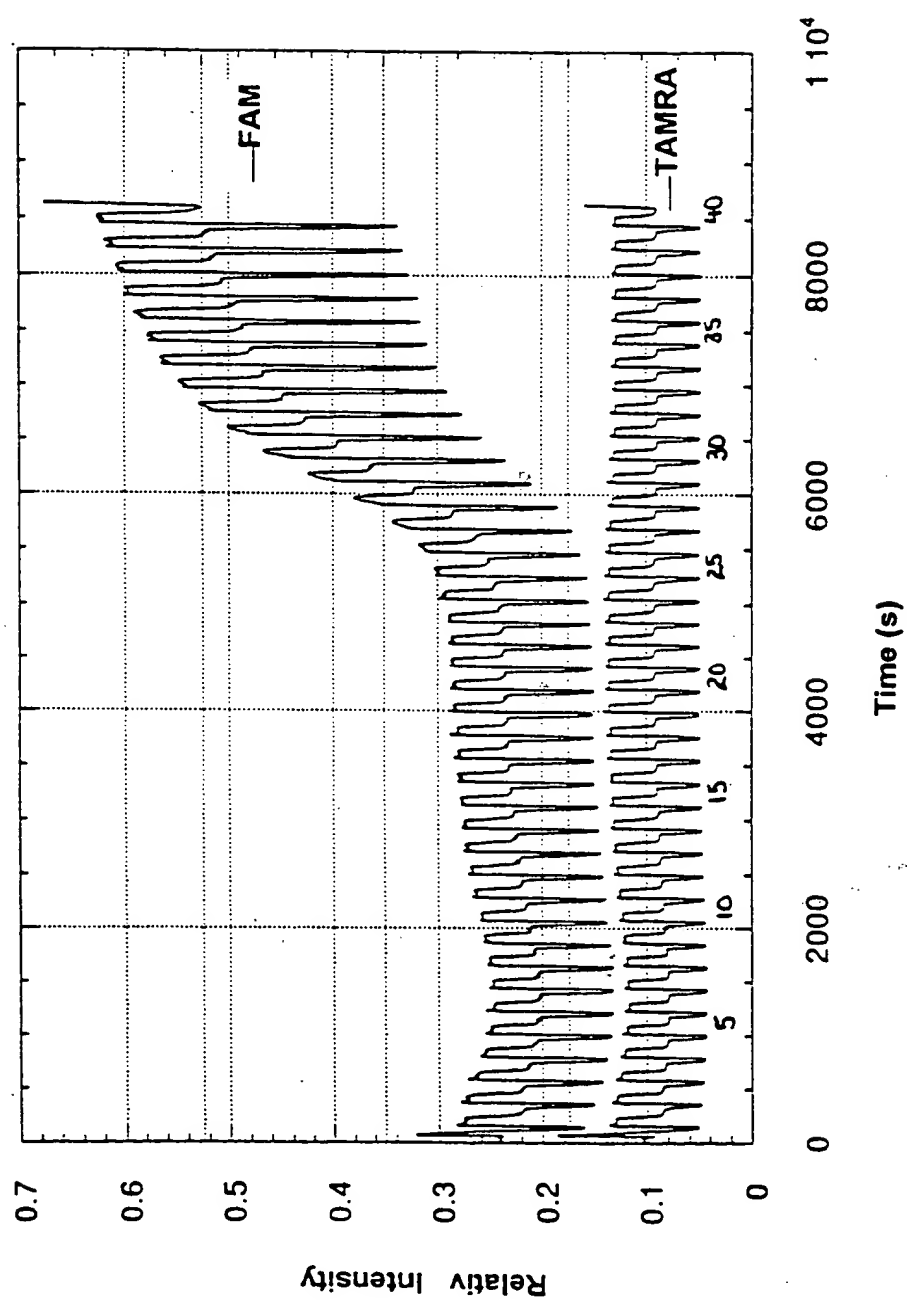**SUBSTITUTE SHEET (RULE 26)**

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   G01N21/64   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOTECHNOLOGY, vol. 10, April 1992 NEW YORK US, pages 413-417, R. HUGUCHI 'Simultaneous amplification and detection of specific DNA sequences' cited in the application see page 415, left column, line 14 - line 17 see page 416, right column, line 62 - page 417, left column, line 3 ---	1-3,8
Y	US,A,4 954 435 (KRAUTH) 4 September 1990 see abstract see column 3, line 16 - line 50 --- -/--	1-3,8

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 1995	- 2. 08. 95

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Thomas, R.M.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 421 156 (PACKARD INSTRUMENT) 10 April 1991 see column 5, line 57 - column 6, line 12 see column 6, line 17 - line 31 see figure 2 ---	1-3,8
A	EP,A,0 108 524 (DYNATECH) 16 May 1984 see abstract see page 10, line 20 - line 26 see page 11, line 18 - page 12, line 5 see page 12, line 26 - page 13, line 2 see figure 4 ---	1
A	US,A,4 916 060 (WEAVER) 10 April 1990 see abstract see column 4, line 55 - column 5, line 9 see column 6, line 61 - column 7, line 1 ---	1
A	WO,A,89 04302 (IGEN) 18 May 1989 see page 85, last paragraph - page 86, line 3 see page 87, last paragraph - page 88, last line see figures 2,3 -----	1

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